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Comparison of immunofluorescence assay and immunomagnetic electrochemiluminescence in detection of *Cryptosporidium parvum* oocysts in karst water samples

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Abstract

Immunofluorescence assay (IFA) and immunomagnetic electrochemiluminescence (IM-ECL) were used for comparison of the percent recovery of *Cryptosporidium parvum* in environmental water samples obtained from a spring draining a karst basin. The monoclonal antibodies to *C. parvum*, isotype IgG3 were used for optimization of the IM-ECL protocol. The combination of biotinylated and TAG-labeled anti-*C. parvum* antibodies with the streptavidin beads gave a linear regression slope for log ECL vs. log fresh oocysts of 0.79 (from 5 to 5000 oocysts), which indicates a constant ECL signal per oocyst. Standard curves gave a dynamic range of 5 to 5000 oocysts/ml (fresh) and 10 to 100,000 cells/ml (4-month-old oocysts) with the maximum limit of linear detection higher than 100,000. The linear slope of 4-month-old oocysts decreased to 0.62, which indicates that ECL signal is a function of oocyst age. The experiment associated with bead storage time shows that even after 4 months of storage of the biotinylated antibodies, the complex retains the ability for binding the oocysts and generating the ECL signal. Based on the IFA results in the experiment evaluating different protocols for oocysts recovery from karst water samples, the most efficient protocol involved dispersion, followed by flotation and immunomagnetic separation (IMS) (24% recovery). The ECL results obtained in that experiment were very similar to the results obtained in the IFA method, which indicates that the IM-ECL method is accurate. Results of the IFA in the study of the prevalence of *C. parvum* in the groundwater showed that oocysts were present in 78% of 1 L water samples with average number of oocysts of 6.4 ± 5.5 and ranged from 0 (13 samples) to 23.3 (2 samples). The ECL signal generated from these water samples ranged from 3771 to 622 (average 1620 ± 465). However, the background value estimated in groundwater samples with low number of oocysts detected by IFA was highly variable and elevated (from 3702 to 272, average 1503 ± 475). The background value as a result of nonspecific binding to beads by unidentified organic components in the water can inhibit or even completely mask the signal generated by oocysts. Our investigations showed that the IM-ECL method appears to be promising for the qualitative and quantitative detection of *C. parvum* from the environmental water; however, the method requires further development to improve sensitivity and account for background signals.
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Keywords: *Cryptosporidium*; Detection; IFA; Electrochemiluminescence; Karst water

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1. Introduction

Cryptosporidium parvum is a widespread, coccidian parasite, which causes gastrointestinal illness in humans and numerous domestic and wild animals. With a low infectious dose, about 30 oocysts (DuPont et al., 1995), this parasite can cause life-threatening infection especially for immunocompromised people (e.g. cancer patients, individuals with diabetes, transplant recipients, AIDS patients), the malnourished, the elderly and infants (Fayer et al., 1998). The mortality rate of cryptosporidiosis in the immunocompromised individuals reaches 50% (Addiss et al., 1995; Juranek et al., 1995). In the past decade, *C. parvum* caused several waterborne outbreaks. Since the massive outbreak in Milwaukee, WI in 1993, where more than 400,000 people were infected (MacKenzie et al., 1994), cryptosporidiosis was noticed in several locations, e.g. in Las Vegas, NV (Roefer et al., 1996), Spokane WA (CDC, 1998), Delaware, OH and Douglas County, NE (CDC, 2001). Most of these outbreaks and cases were connected with contamination of water supplies or recreational water. *C. parvum* oocysts, resistant to standard chlorination disinfection, can survive in aquatic environments for several months (Robertson et al., 1992). Many surface waters are often contaminated by oocysts from agricultural runoff due to infected livestock, sewage effluent and wildlife (LeChevallier et al., 1991a, 1991b). However, the major contributors of *C. parvum* water contamination are considered to be dairy and beef calves (Garber et al., 1994; Scott et al., 1994). A large outbreak in the United Kingdom, resulting in 55,000 illnesses, was caused by rainfall washing oocysts from infected cattle into the water supply (Smith and Rose, 1990).

In addition to the surface mode of transport of oocysts to potable or recreational water, it is also possible that oocysts attached to small particles can leach into groundwater through macroporous soils, bedrock fractures and karst conduits. Due to known constant interaction between these factors and water quality, *Cryptosporidium* remains a serious concern to the water industry. The EPA developed a comprehensive nationwide monitoring program termed the Information Collection Rule (ICR) in 1996, which requires routine monitoring for these protozoa. (USEPA, 1996).

To minimize the impact of this parasite on environmental water, an accurate procedure is required to determine the oocysts' presence and its enumeration. Although there are many methods known for detection of *C. parvum* in water, they are labor-intensive, time-consuming and generally have low and variable efficiencies (Rose et al., 1989; Clancy et al., 1994; Nieminski et al., 1995). Most of them are based on the concentration by retention filtration or centrifugation and subsequent immunofluorescence techniques. However, the presence of particulate material, which is accumulated during the concentration, often interferes with the microscopic analysis. In addition, other particles of similar size and density (e.g. algae) may also interfere with the examination of the concentrate by epifluorescence microscopy (Clancy et al., 1994; Rodgers et al., 1995). Usually the debris is removed by the use of various density flotation procedures including sucrose, Percoll, cesium chloride or salt solution. However, those methods often reduce the amount of oocysts along with the interfering material.

Various alternative methods, based on the immunological technologies, have been investigated in efforts to provide more effective detection of waterborne *Cryptosporidium*. These include flow cytometry (Vesey et al., 1994; Arrowood et al., 1995), enzyme-linked immunosorbent assays (De la Cruz and Sivaganesan, 1994), polymerase chain reaction (Johnson et al., 1995; Rochelle et al., 1997) and immunomagnetic separation (IMS) (Fricker et al., 1997; Rochelle et al., 1999; Pereira et al., 1999; McCuin et al., 2001), which has recently become commercially available. IMS can selectively remove oocysts from other debris based on the specificity of monoclonal antibodies. The method utilizes antibodies linked to the surface of magnetic particles to attach to oocysts, which are selectively concentrated from surrounding matter by using magnetic fields. Campbell et al. (1997) reported over 60% recovery of *C. parvum* oocysts from high turbidity, 5000 to 10,000 nephelometric turbidity unit (NTU) water. Bukhari et al. (1998) obtained similar results in water matrices with turbidity level of 5000 NTU. Oocyst recoveries ranged from 35% to 70% in that study.

Recently, methods utilizing IMS in conjunction with electrochemiluminescence (IM-ECL) for labeling and detection of bacterial and protozoan pathogens have been described. Yu and Bruno (1996) first reported an IM-ECL based protocol for the qualitative detection of *E. coli* O157 and *Salmonella typhimurium*

in various foods and water samples. Shelton and Karns (2001) developed and optimized an IM-ECL protocol for the quantitative detection of *E. coli* O157 in raw and concentrated surface water samples. Lee et al. (2001) reported the development of an IM-ECL protocol for detection of solubilized *C. parvum* oocysts in water samples. However, they did not compare results derived from the IM-ECL protocol with traditional immunofluorescence assay (IFA) procedures.

The objective of the present study was to compare an IM-ECL protocol with standard IFA procedures for the quantitative detection of intact *C. parvum* oocysts in karst groundwater samples from the Appalachian region. The impact on water quality by agricultural activity in karst terrain is an important consideration for resource management within the Appalachian Region. Karst areas comprise about 18% of the Appalachian region's land area (Davies, 1984). An estimated one-third of the Region's farms, cattle and agricultural market value are located on karst terrain (Boyer and Pasquarell, 1995). An understanding about the extent and transport characteristics of *C. parvum* in karst aquifers is essential to protection of drinking water for small rural communities, farm families and livestock in the region.

2. Materials and methods

2.1. Oocyst standards

Purified *C. parvum* oocysts (ca. 10^7 cells ml^{-1}) were obtained from Immunology and Disease Resistance Laboratory (Beltsville Agricultural Research Center, Maryland). The isolate was obtained from infected calves, as previously described (Fayer et al., 1998) and was stored in sterile phosphate-buffered saline (PBS; pH 7.2) at 4 °C until use. The age of oocysts used in these experiments was approximately 1 month, unless otherwise specified. Precise numbers of oocysts were determined with a Neubauer hemocytometer using phase contrast microscopy (250 ×; average of six determinations). Stock solution suspension was diluted to serial concentrations 5, 10, 50, 100, and 1000 ml^{-1} oocysts with PBS. Suspensions with low numbers of oocysts were enumerated by placing ten 10-μl replicate aliquots on glass well microscope slides. The slides were then dried on a slide warmer and stained using a commercial immunofluorescence (IFA) kit (Merifluor, Meridian Diagnostic Cincinnati, OH, USA) and examined with an epifluorescence microscope (Zeiss) using × 250 magnification.

2.2. Water sample collection and preparation

Fifty-nine water samples (10 l each) were collected from a 14.5-km^2 karstic agricultural watershed outlet spring during base flow conditions. The study watershed, located in southeastern West Virginia USA, is called The Hole Basin and is completely drained internally to a spring called Burns' Cave #2. The watershed and spring were previously described by Boyer and Pasquarell (1995). All samples were concentrated by centrifugation (1500 × *g*, 10 min each cycle) in 700-ml polypropylene bottles. The final sediment was resuspended in PBS and transferred to the 15-ml polypropylene tubes until use.

Following this procedure, the PBS from all samples was decanted, the dispersion solution (50 mM Tris and 0.5% Tween 80 v/v) was added and the samples were vigorously vortexed for 3 min and centrifuged at 1000 × *g* for 10 min. After decanting the supernatant, the oocysts were separated from the sediment by using the NaCl flotation method, as previously described by Kuczynska and Shelton (1999). The tubes were centrifuged at 1000 × *g* for 10 min, the recovered supernatant was washed 3 × with PBS by repeating centrifugation and finally concentrated to 100 μl. Microscope slides were prepared from each sample, stained using the IFA kit (Merifluor) and examined in the method described in the section of Oocyst Standards. The remaining 70 μl from each sample was then transferred to an ECL assay tube and suspended in 5 ml of phosphate-buffered saline-2 (PBS-2) composed of 150 mM potassium phosphate buffer (pH 7.2), 150 mM NaCl and 0.1% sodium azide.

2.3. Antibodies, beads and IM-ECL procedure

Commercial 2.8-μm streptavidin-coated beads—Dynabeads M-280™ (Dynal, Oslo Norway)—were purchased from IGEN International (Gaithersburg, MD). Biotin-LC-sulfoNHS ester and ORIGEN TAG-NHS™ ester [(ruthenium(ii) tris-bipyridyl [Ru(bpy3]

(referred to hereafter as TAG)] were purchased from IGEN International). The monoclonal antibodies to *C. parvum* (2 mg of MAb CP107), isotype IgG3, were obtained as a purified liquid from BioDesign International (Saco, Maine). Desalinization of the antibodies was accomplished by a gel-filtration column NAP-5 (Pharmacia Biotech), equilibrated preliminarily with 150 mM PBS-1. The biotinylation and TAG-labeling of antibodies were conducted according to the method previously described by Shelton and Karns (2001). The attachment of the biotinylated antibodies to the beads (BMAb) was conducted immediately prior to use, except in the experiment associated with the examination of the bead storage time. In this experiment, the complex of biotinylated MAb and beads was stored at 4 °C for 2 and 4 months.

The streptavidin beads (0.2 mg/ml) and biotinylated MAb (0.5 μg/ml), in 50-μl aliquots for each assay tube, were incubated by shaking for 1 h. To capture *C. parvum* oocysts, the complex BMAb (100 ml) was added to the sample suspended in 5 ml of PBS-2 and rotated overnight using rotamixer RKVSD (Appropriate Technical Resources Laurel, MD). Beads were recovered for 15 min using MPC-S magnetic particle concentrator (Dynal). After discarding the supernatant, the isolated beads with captured oocysts were suspended in 200 μl of PBS-2. TAG-antibodies, 50 μl (2 μg/ml), were added to each tube and the mixture was incubated shaking for 3 h, using Gyrotory Shaker-Model G2 (New Brunswick Scientific Edison, NJ). The total volume was 250 μl. Assay standards were prepared (three replicates of each concentration of oocysts) as well as triplicates of PBS-2 blanks (negative control), which were run at the beginning and the end of each standard curve. Samples were analyzed using the ORIGEN™ manufactured by IGEN International with the method described by Shelton and Karns (2001). Adjustable instrument parameters were: assay gain 100, instrument background subtracted and signal averaged. Net ECL unit was calculated by subtracting the signal generated by PBS-2 blanks from the ECL signal generated by samples.

2.4. Methods of debris removal

To determine the capture efficiency for *C. parvum* oocysts, four different cleaning methods were compared using about 5 ml of sediment collected from the study spring. After microscopic examination, using the IFA method, the absence of *C. parvum* oocysts was determined; the sediment was divided into 48 100-μl samples and all samples were resuspended in 1 ml of distilled water. Each sample was spiked with 100 fresh *C. parvum* oocysts. The seeded samples were divided into four groups, with 12 repetitions in each, and treated in four different methods.

After decanting the water from samples in the first group (A), they were treated with dispersion solution (50 mM Tris and 0.5% Tween 80 v/v) and the NaCl flotation method was performed. The remaining pellet of each sample was resuspended in 5 ml of PBS, transferred to the ECL assay tubes and 100 μl of the prior prepared complex BMAb (streptavidin beads (0.2 mg/ml) and biotinylated Mab (0.5 μg/ml) was added, in 50-μl aliquots). After overnight rotation using the rotamixer, beads were captured and samples were prepared for the ORIGEN™ analyzer using the method described in Section 2.3.

The second group (B) of 12 samples was cleaned using NaCl flotation and one overnight bead capture. The sediment of the next 12 samples (group C) was resuspended in 5 ml of dispersion solution (50 mM Tris and 0.5% Tween 80 v/v), then 130 μl of the complex BMAb was added and the bead capture was performed two times. In the fourth group (D) 130 μl of the complex BMAb was also added to the samples, but the bead capture was carried out three times.

Microscope slides were prepared from each sample (A and B group—after flotation; C and D group—after the last bead capture), stained using the IFA kit and examined using the epifluorescence microscope. After suspension in 200 μl of PBS-2, each sample was prepared and analyzed using the ORIGEN™ in the method described in Section 2.3.

2.5. Statistics

Statistical analyses were performed with the Statistical Analysis System (SAS™). The general linear models (GLM) procedure was used to calculate regression statistics. Statistical comparisons of means were made with the analysis of variance (ANOVA) procedure and the least-significant-difference test.

Table 1
Comparison of *C. parvum* oocysts recovery by ECL assay ($n=9$) using IM beads freshly prepared, stored for 2 months, and stored for 4 months

Oocysts (ml^{-1})	Mean net ECL signal ($\pm$ S.D.)		
	Fresh	2 months	4 months
5	162 ± 22^{A}	147 ± 43^{A}	107 ± 25^{B}
10	385 ± 20^{A}	374 ± 28^{A}	282 ± 47^{B}
100	1993 ± 114^{A}	1825 ± 139^{B}	1203 ± 178^{C}
1000	7746 ± 702^{A}	6318 ± 352^{B}	6220 ± 912^{B}

Mean ECL signals with the same letter (A, B and C) in a row are not significantly different at the $P=95\%$ level.

Statistical significances are reported at the 95% probability level unless stated otherwise.

3. Results

3.1. Assay optimization and evaluation

Preliminary experiments indicated that the optimal concentration for biotinylated antibodies is 0.5 μg/ml, streptavidin beads, 0.2 mg/ml, and 2 μg/ml TAG-labeled antibodies. Also, the optimal incubation time for TAG exposure to the biotinylated Mab–streptavidin beads–oocysts complex was 3 h.

The experiment associated with bead storage time was conducted using four concentrations of 1-month-old *C. parvum* at 5, 10, 100 and 1000 oocysts ml^{-1} as standards. The biotinylated monoclonal antibodies attached to the immunomagnetic beads (BMAb) were stored for 2 to 4 months. The results were calculated from 9 replicates of each oocyst concentration and triplicate PBS-2 blanks. The net ECL signals are presented in Table 1. These data show that even after 4-month storage of the BMAb the complex retains the ability for binding the oocysts and generating the ECL signal. The signal decreases from that for freshly prepared beads averaged 9.8% (range 2.9% at 10 oocysts ml^{-1} to 18.4% at 1000 oocysts ml^{-1}) and 30% (range 19.7% at 1000 oocysts ml^{-1} to 39.6% at 100 oocysts ml^{-1}) for beads prepared 2 and 4 months prior to use, respectively.

For quantitative detection of *C. parvum* using ECL method, oocysts approximately 1 and 4 months old were used (Fig. 1). Each assay was performed using six replicates of the standard oocysts concentrations. Standard curves gave a dynamic range of 5 to 5000 oocysts ml^{-1} (fresh) and 10 to 100,000 cells ml^{-1} (4-month-old oocysts). The value of net ECL unit for 100,000 oocysts (44,066) indicates that the maximum limit of linear detection is higher than 100,000. The linear regression slope for log ECL vs. log fresh

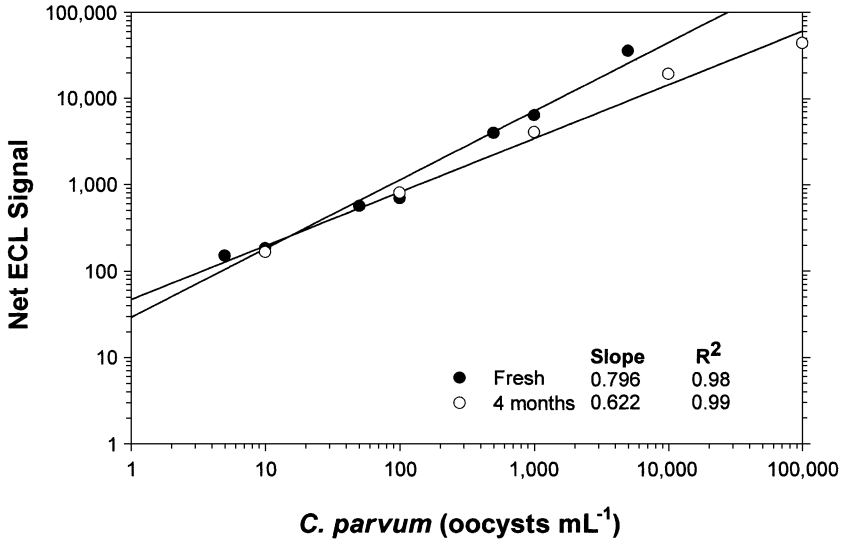

Fig. 1. Standard curves for 1- and 4-month-old *C. parvum* oocysts in PBS2 ($n=6$). Linear regression slopes were computed using concentrations of 5, 10, 50, 100 and 1000 of fresh, and 10, 100, 1000, 10,000 and 100,000 of 4-month-old oocysts ml^{-1}.

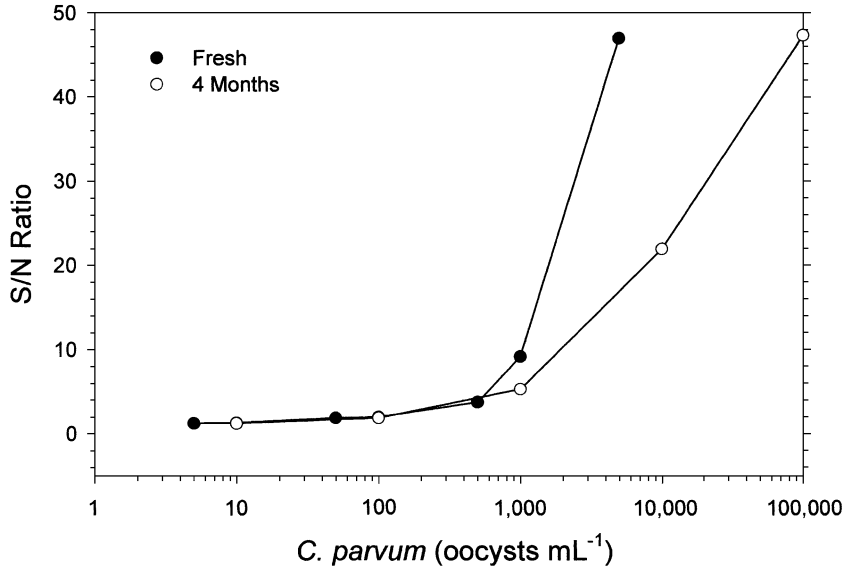

Fig. 2. Evaluation of the signal/noise ratios (S/N) from different concentrations of *C. parvum* oocysts in PBS2 for 1- and 4-month-old oocysts; S/N values were calculated by dividing the ECL signal generated from oocysts standards by the signal generated from PBS2 blanks.

oocysts was 0.796 (P>99%) while for log 4-month oocysts the slope decreased to 0.622 (P>99%).

Signal-to-background noise ratio (S/N) (Fig. 2) was obtained by dividing the net ECL signal generated from standards containing different concentrations of oocysts by the net ECL signal generated from PBS-2 blanks (negative control). The S/N ratio generated by standards containing 1-month-old oocysts of *C. parvum* was slightly higher than the ratio generated by 4-month-old oocysts at concentrations of 1000 oocysts ml^{-1} and lower. The difference was increasingly greater at concentrations greater than 1000 oocysts ml^{-1}. The lowest S/N ratio (less than 2), as well as the S/N ratio for fresh and old oocysts, was obtained from standards with concentrations of 5 to 100 oocysts and the ratio increased together with highest number of *C. parvum* to 46.98 for fresh (concentration 5000) and 47.32 for 4-month-old (concentration 100,000) oocysts.

3.2. Evaluation of oocysts recovery protocols

The evaluation of four methods for cleaning the environmental water sediment was based on the comparison of the microscopy and ECL assay results (Table 2). The percentages of the oocyst recoveries using IM-ECL were slightly higher than using IFA and ranged from 15 to 29 and 11 to 24, respectively. The highest recovery of oocysts using IFA kit (24%) was obtained with dispersion solution followed by

Table 2
Comparison of *C. parvum* oocyst recoveries using different extraction procedures and IM-ECL vs. IFA detection methods

Extraction procedure	ECL		IFA
	Net ECL signal (± S.D.)[a]	Percent recovery[b]	Percent oocysts recovered (± S.D.)[a]
A[c]	206 ± 49	28	24 ± 6
B[d]	110 ± 26	15	11 ± 5
C[e]	123 ± 38	16	14 ± 8
D[f]	218 ± 126	29	20 ± 7

[a] Water spiked with 100 oocysts (n = 12).
[b] Based on ca. 7.5 ECL units $oocyst^{-1}$; derived from standard curve.
[c] Dispersion, flotation, and bead capture.
[d] Flotation and one bead capture (1 × washing).
[e] Dispersion and bead capture (2 × washing).
[f] Bead capture only (3 × washing).

flotation and one bead capture (procedure A). Slightly lower microscopy results (20%) were obtained from samples after three sequential beads capture (procedure D). Procedure D resulted in the highest mean net ECL signal (218), however, the standard deviation was more than twice as high as procedure A. The mean net ECL signal obtained from procedure A (206) was similar to the mean net ECL signal from procedure D (218). The lowest recovery of *C. parvum* oocysts using IFA kit and IM-ECL analyzer was obtained from samples treated with flotation and one bead capture (procedure B). Within each detection method there was no significant difference between procedure A and D or between B and C.

3.3. Analysis of water samples

The study of the prevalence of *C. parvum* oocysts in the groundwater were conducted from the beginning of January to the end of December 2000, using the IFA kit and ECL analyzer (Fig. 3). Results of the immunofluorescence microscopy showed that *C. parvum* oocysts were present in 78% of water samples. The average number of oocysts detected in 10-l samples was 6.4 ± 5.5 and ranged from 0 (13 samples) to 23.3 (2 samples). The highest number of oocysts was detected in May.

The ECL signal generated from water samples ranged from 3771 to 622 (average 1620 ± 465) (Fig. 3b). The background signal was estimated by sub-

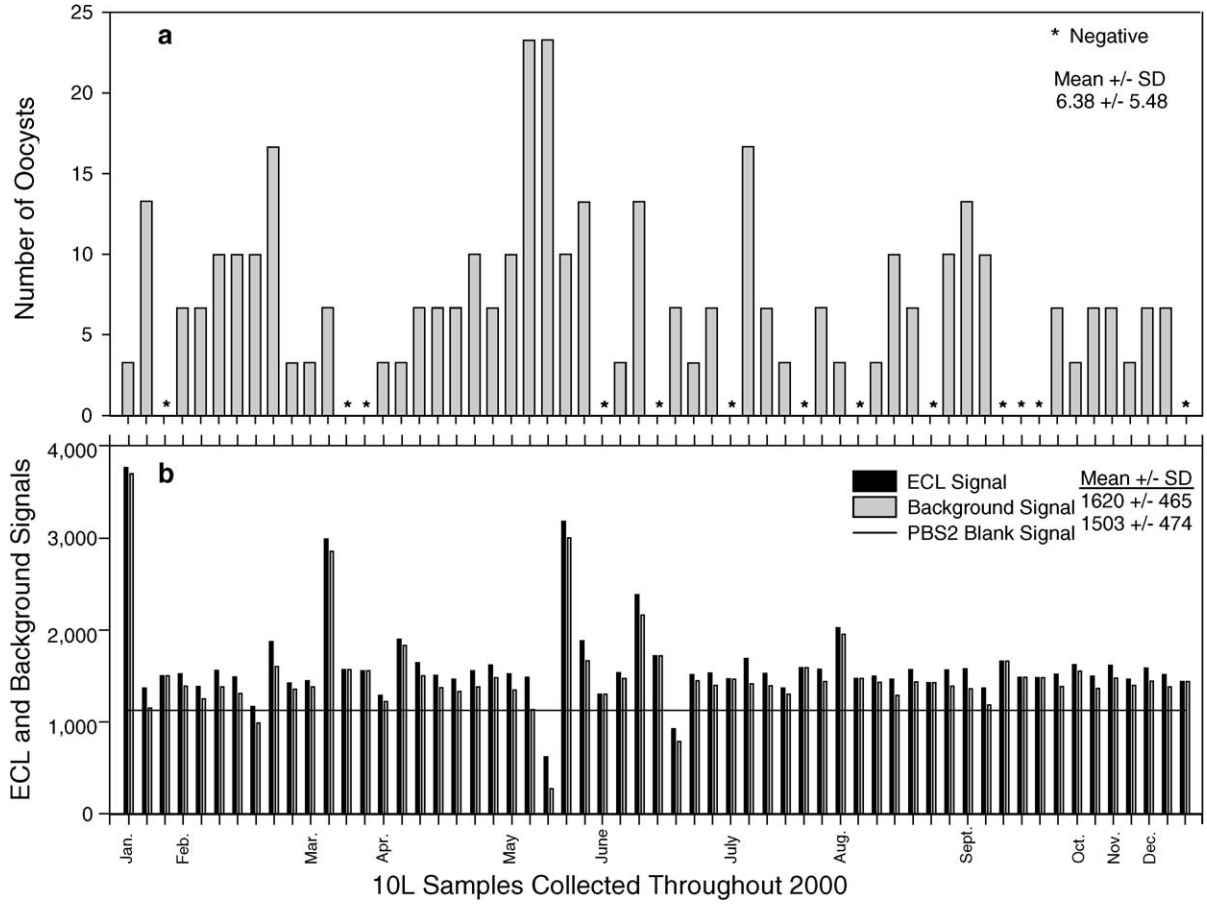

Fig. 3. Detection of *C. parvum* oocysts in 10 l of groundwater samples collected from a karst watershed in Appalachian region using IFA kit (a) and IM-ECL method (b). Background signal was calculated by subtracting from ECL signal the estimated ECL signal according to the standard curve for specific amount of oocysts determined by IFA.

tracting ECL signal according to the standard curve for the specific amount of oocysts detected by IFA from the ECL signal generated from the appropriate sample. The background signal ranged from 3702 to 272 (average 1503 ± 475). Two samples, one collected in May and the other in June, generated lower ECL signals than the average signal obtained from PBS-2 blanks (1132) although the number of oocysts detected by IFA was 23 and 7, respectively. For 13 samples containing no oocysts according to IFA, the ECL signal ranged from 1303 to 1722 with an average of 1517 ± 107.

4. Discussion

A linear relationship was observed between the ECL signal and *C. parvum* oocyst concentration, although the magnitude of the signal decreased with oocyst age. The slope with fresh oocysts was 0.79 vs. 0.62 for 4-month-old oocysts. Presumably, the ECL signal per oocyst would continue to decrease with age, although the precise relationship was not determined.

These data suggest that oocysts lose epitopes with age, although this does not necessarily affect capture efficiency. Bukhari et al. (1998) did not observe significant differences in IM bead capture and detection of fresh and 6- to 16-week-old "aged" oocysts. McCuin et al. (2001) obtained similar results using 6-month-old oocysts for spiking water samples: 81.4% of recovery for samples processed after a few hours and 68.6% for samples processed after 20 months. However, the binding of antibody-TAG would diminish, affecting ECL signal. Since the age of oocysts in environmental water samples is unknown, and likely to be variable, IM-ECL can provide only a semi-quantitative value for oocyst concentrations.

In our study we did not observe significant differences in the ability of the 2-month-old IM beads vs. fresh beads to bind low numbers (5 and 10) of oocysts, although ECL values for 100 and 1000 oocysts were slightly lower for 2-month-old IM beads. This could be very convenient for examiners, especially in commercial laboratories, to prepare large volumes of beads at once, and store them between 0 and 8 °C, thereby eliminating the need for bead preparation every time water samples are analyzed.

Environmental water samples contain varying amounts of sediment, composed of some soil and mineral particles, colloids, and other organic matter. Because *C. parvum* oocysts adhere to particulate matter, detection and quantitation is dependant on detachment of the oocysts from the particulate matter. All of our procedures used for debris removal resulted in losses of oocysts, from 75% to 89%. Based on the IFA results, the most efficient method of recovering oocysts involved dispersion, followed by flotation and immunomagnetic separation (24% recovery). Note that the ECL signals were generally consistent with IFA counts, regardless of extraction procedure.

Karst water samples (10 l) were concentrated by centrifugation and treated with dispersion solution followed by flotation and bead capture. These samples contained approximately 0.1 to 0.5 ml of sediment. Based on IFA, *C. parvum* oocysts were ubiquitous in karst groundwater, although at low density (6.4 ± 5.5). Based on the percentage of oocysts recovery (24%), the actual mean number of *C. parvum* is ca. 25 oocysts $10\ l^{-1}$. This may still be an underestimate due to several factors. Groundwater quality may have an impact on the method of recovery. Karst groundwaters frequently have a high mineral content, for example lime (calcium hydroxide), which is positively charged and may play an active role in lowering recovery of organisms with negative zeta potential like *Cryptosporidium* and *Giardia* (Klonicki et al., 1996). Also the iron content in groundwater may interfere with the immunomagnetic separation method.

Based on standard curves, it is theoretically possible to detect such small numbers of oocysts. However, this assumes a consistent background signal. We did not observe a correlation between the ECL signal and oocysts concentrations in the karst water samples due to high background signals. The background values from groundwater samples with low number of oocysts detected by IFA were highly variable and elevated. These results suggest that the background values were a result of nonspecific binding to beads by unidentified organic components in water. The background value can inhibit or even completely mask the signal generated by the oocysts. These data indicate that additional procedures may be necessary to establish background baseline values. For example, Shelton and Karns (2001) have described a procedure,

termed sequential bead capture (SBC), to establish baseline ECL values for individual water samples.

Our results are generally comparable to the results obtained by Lee et al. (2001), although there are some important differences. In their study a linear relationship between oocyst concentration and ECL signal was not observed. According to their statement the protocol could be used to quantify oocysts in environmental samples, but a standard curve was not included in their results. Also, it was observed that fish gelatin enhanced the ECL signal in high turbidity samples containing clay-rich soil. However, turbidity per se is not indicative of background binding, because nonspecific background binding is primarily due to organic compounds rather than minerals. Consequently, it is unclear what their threshold of detection would be in environmental water samples containing variable concentrations of organic compounds. Finally, it is uncertain whether the IM-ECL results were consistent with any other *Cryptosporidium* oocysts detection method, because no validation study was conducted.

Lee et al. (2001) solubilized oocyst walls prior to IM-ECL analysis, thereby increasing the concentration of epitopes. Although solubilization should theoretically be more sensitive, there are several advantages of using the IM-ECL method with intact oocysts. After capture on IM beads, intact oocysts can be (i) counted using IFA/microscopy data, (ii) speciated or genotyped, and (iii) analyzed for viability.

In conclusion, our investigations showed that the IM-ECL method appears to be promising for the qualitative and semi-quantitative detection of *C. parvum* from the environmental water. However, the method requires further development to improve sensitivity and minimize nonspecific background binding.

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